European Journal of Pharmacology 734 (2014) 122–131

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

Cytoprotection of human endothelial cells against oxidative stress by 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im): Application of systems biology to understand the mechanism of action

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ARTICLE INFO

Article history:
Received 13 December 2013
Received in revised form
18 March 2014
Accepted 24 March 2014
Available online 3 April 2014

Keywords:
CDDO-Im
CAPE
Cytoprotection
Oxidative stress
Heme oxygenase-1
Gene expression analysis

ABSTRACT

Cellular damage from oxidative stress, in particular following ischemic injury, occurs during heart attack, stroke, or traumatic injury, and is potentially reducible with appropriate drug treatment. We previously reported that caffeic acid phenethyl ester (CAPE), a plant-derived polyphenolic compound, protected human umbilical vein endothelial cells (HUVEC) from menadione-induced oxidative stress and that this cytoprotective effect was correlated with the capacity to induce heme oxygenase-1 (HMOX1) and its protein product, a phase II cytoprotective enzyme. To further improve this cytoprotective effect, we studied a synthetic triterpenoid, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), which is known as a potent phase II enzyme inducer with antitumor and anti-inflammatory activities, and compared it to CAPE. CDDO-Im at 200 nM provided more protection to HUVEC against oxidative stress than 20 μM CAPE. We explored the mechanism of CDDO-Im cytoprotection with gene expression profiling and pathway analysis and compared to that of CAPE. In addition to potent up-regulation of HMOX1, heat shock proteins (HSP) were also found to be highly induced by CDDO-Im in HUVEC. Pathway analysis results showed that transcription factor Nrf2-mediated oxidative stress response was among the top canonical pathways commonly activated by both CDDO-Im and CAPE. Compared to CAPE, CDDO-Im up-regulated more HSP and some of them to a much higher extent. In addition, CDDO-Im treatment affected Nrf2 pathway more significantly. These findings may provide an explanation why CDDO-Im is a more potent cytoprotectant than CAPE against oxidative stress in HUVEC.

1. Introduction

Mammalian cells are constantly exposed to reactive oxygen species generated from oxidase systems, mitochondria metabolism, and external sources (e.g. UV irradiation, xenobiotics) (Finkel, 2012; Ma, 2010). Reactive oxygen species at low concentration serve as important signaling messengers in the processes of cell division, inflammation, and stress response (Finkel, 2011). Under normal conditions, cells can neutralize extra reactive oxygen species through redox reactions with intracellular antioxidants. However, sustained production of oxidants (pro-oxidants or reactive oxygen species) more than cellular reductants (antioxidants) can counterbalance, leads to oxidative stress. The oxidative damage generated by overproduction of reactive oxygen species exacerbates cardiovascular diseases, cancer, diabetes, chronic inflammation, stroke, septic shock, and neurodegenerative diseases (Heitzer et al., 2001; Ma, 2010). In addition, the theory of free radicals remains a major contributor to aging (Halliwell and Gutteridge, 2006).

In a continuing effort to find new ways to ameliorate oxidative stress-mediated ischemia–reperfusion injury, we identified a natural polyphenolic compound, caffeic acid phenethyl ester (CAPE), as a potential cytoprotectant. Our previous studies showed that CAPE protected human umbilical vein endothelial cells (HUVEC) against menadione-induced oxidative stress and that this protection was largely due to induction of a cytoprotective gene, heme oxygenase-1 (HMOX1), by CAPE (Wang et al., 2010, 2008). The protein product of HMOX1, heme oxygenase-1 (HO-1), usually known to be the rate-limiting enzyme for heme degradation, is now considered as

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http://dx.doi.org/10.1016/j.ejphar.2014.03.033
0014-2999/

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
02 APR 2014	**N/A**	**-**

4. TITLE AND SUBTITLE

Cytoprotection of human endothelial cells against oxidative stress by 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im): Application of systems biology to understand the mechanism of action

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Wang X., Bynum J. A., Stavchansky S., Bowman P. D.,

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

US Army Institute of Surgical Research, JBSA Fort Sam Houston, TX

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**10**	

a phase 2 detoxification enzyme (Talalay, 2000). To further improve cytoprotection of human endothelial cells, we investigated a recently described inducer of phase 2 enzymes, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), a new synthetic triterpenoid (Place et al., 2003).

CDDO-Im is the imidazolide derivative of its parent compound, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO). CDDO, a synthetic oleanane triterpenoid, was identified through an attempt to design new anti-inflammatory agents from the natural products oleanolic acid and ursolic acid (Honda et al., 1998; Suh et al., 1999). CDDO, CDDO-Im, and methyl ester derivative of CDDO also showed antitumor activities in vitro and in animals (Ito et al., 2000; Kim et al., 2002; Place et al., 2003). A recent study found that CDDO and CDDO-Im induced HO-1 both in vitro and in vivo, and the imidazolide derivative was more potent (Liby et al., 2005). This induction was possibly through the activation of a signaling pathway regulated by transcriptional factor nuclear factor, erythroid 2-like 2 (Nrf2). Upon activation, Nrf2 translocates from the cytoplasm to the nucleus and binds to the antioxidant responsive element (ARE) to initiate transcription of an array of drug metabolism and antioxidant genes. Activation of this Nrf2/ARE pathway leads to an increased elimination of xenobiotics and thus increased resistance to oxidative stress (Ma, 2013).

The purpose of our present research was to (1) identify more potent cytoprotectants against oxidative stress than CAPE and (2) obtain insight into potential mechanisms of this cytoprotection through a genome-wide systems biology approach. In the study, we examined the cytoprotective effect of CDDO-Im against oxidative stress in HUVEC and compared it to CAPE. A microarray analysis was conducted on HUVEC after a 6-h treatment with either CDDO-Im or CAPE to look for induction of cytoprotective genes. Data were analyzed through the use of Ingenuity Pathway Analysis (IPA) for functional enrichment and pathway analysis of microarray data to identify potential involvement of cellular functions and canonical pathways mediating oxidative stress.

2. Materials and methods

2.1. Chemicals and reagents

CAPE was purchased from Cayman Chemicals (Ann Arbor, MI, USA). CDDO-Im was kindly provided by Dr. Michael Sporn (Dartmouth Medical School, Hanover, NH, USA). Menadione sodium bisulfite (menadione) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

2.2. Cell culture

HUVEC (Life Technologies, Carlsbad, CA, USA) were cultivated on 1% gelatin-coated 75 cm^2 culture flasks (Corning Incorporated, Corning, NY, USA) in Medium 200 supplemented with 2% fetal calf serum, penicillin (100 units/ml), streptomycin (100 units/ml), and fungizone (0.25 μg/ml) supplied by Life Technologies (Wang et al., 2008). Stock cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 with medium changes every 2 days till confluent. Prior to an experiment, HUVEC were subcultivated with trypsin/EDTA onto 1% gelatin-coated 48-or 96-well Costar® multiplates (Corning Incorporated) at 10,000 or 5000 cells/well, respectively, grown to confluence, and kept for 72 h to produce a quiescent cell layer. On the day prior to the experiment, the medium was changed. Only the second through fifth population doubling levels of cells were used.

2.3. Cell viability and toxicity assay

Cell viability was estimated in HUVEC using Alamar Blue™ (Life Technologies), which utilizes metabolic conversion of resazurin to fluorescent resorufin by viable cells. As previously described (Wang et al., 2008), menadione was used to induce oxidative injury in HUVEC. Briefly, HUVEC were exposed to a series of doses of menadione for 24 h. The cells were incubated with culture medium containing 10% Alamar Blue™ for an additional 2 h. Fluorescence was measured at 545 nm excitation and 590 nm emission with a SpectraMAX® M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA). A dose of menadione close to its maximum toxicity, causing 80–90% cell death, was chosen for the cytoprotection assay. Nontoxic doses of CDDO-Im in HUVEC were determined as follows. A series of doses of CDDO-Im (50–1000 nM) were incubated with HUVEC for 24 h followed by cell viability assay. Doses of CDDO-Im causing more than 90% cell viability were considered not toxic to HUVEC and used in the following cytoprotection assay.

2.4. Cell protection assay

CDDO-Im and CAPE were dissolved in DMSO and diluted 1000-fold with medium (final concentration of DMSO no more than 0.1%). Confluent HUVEC were pretreated with CDDO-Im and CAPE at nontoxic doses for 6 h. They were then exposed to the preselected dose of menadione for an additional 24 h. Cell viability was measured using the Alamar Blue™ assay.

2.5. mRNA-based microarray expression profiling

HUVEC were incubated with CDDO-Im at 200 nM or CAPE at 20 μM for 6 h. Total RNA was extracted from treated HUVEC grown in 6-well plates with TRI™ reagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH, USA). The amount of isolated RNA samples was quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The integrity of isolated RNA samples was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies. Inc., Santa Clara, CA, USA).

Gene expression profiling of HUVEC treated with CDDO-Im or CAPE versus vehicle control (DMSO, 0.1%) was performed using Agilent 60-mer Whole Human Genome Microarrays (Agilent Technologies). Statistical analysis for differences among treatment groups was performed using BRB Array Tools (Biometric Research Branch, National Cancer Institute, USA, http://linus.nci.nih.gov/BRB-ArrayTools.html). Genes were determined to be statistically altered in their expression with both P value <0.001 and false discovery rate (FDR) <0.001 after class comparison between treated and control groups. In addition, the significant gene lists from either CDDO-Im or CAPE treatment were intersected through BRB Array Tools to identify common genes significantly altered by both compounds.

2.6. Ingenuity pathway analysis

The lists of significant genes from microarray data analysis were submitted to IPA for gene function and canonical pathway analyses (Ingenuity® Systems, www.ingenuity.com). IPA maintains a large knowledge database of modeled relationships among proteins, genes, complexes, cells, tissues, drugs, pathways, and diseases generated from published reports. When a date set containing gene identifiers and corresponding expression values such as fold change was uploaded into IPA application, each gene identifier was mapped to its corresponding gene object in this Ingenuity Pathway Knowledge Base (IPKB). A P value and FDR

cutoff of 0.001 were previously applied to the gene sets before submitting to IPA. A fold change cutoff of 2 for both up- and down-regulated genes was applied. These selected genes, called Focus Genes in IPA, were overlaid onto a global molecular network developed from information contained in the IPKB. Networks of Focus Genes were then generated based on their connectivity. IPA calculated a significant score for each network. The higher the score, the more interconnected among those genes within that network. The scores for networks represent the negative log of the *P* value. Therefore, scores of 2 or higher provide at least 99% confidence of not being generated by chance alone. Genes are represented as single nodes in the network.

A functional analysis of the network was done to identify the biological functions that were most significant to the genes in the network. A right-tailed Fisher's exact test was used to calculate a *P* value to determine the probability that each biological function assigned to that network would be due to chance alone. The smaller the *P* value, the less likely the association is random and the more significant the association. In addition, a functional analysis of the entire data set was done to identify the biological functions that were most significant to the data set. The same right-tailed Fisher's exact test was used to calculate a *P* value to determine the probability that each biological function assigned to the entire data set is random. A canonical pathway analysis of the submitted data set was also performed to identify the pathways from the IPA library of canonical pathways that were most significant to the data set. The significance of the association between the data set and the canonical pathway was also measured using right-tailed Fisher's exact test. The *P* value calculated was used to determine the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone.

2.7. Quantitative real-time RT-PCR

One μg of total RNA from the same samples used for microarray analysis was converted to cDNA using random primers and Superscript III reverse transcriptase according to the manufacture's instruction (Invitrogen™/life Technology, Carlsbad, CA, USA). Real-time PCR was performed on a LightCycler™ thermal cycler (Idaho Technology, Salt Lake City, UT, USA). Roche LightCycler® TaqMan Master was used for reverse-transcription (Roche Diagnostics, Indianapolis, IN, USA) and HMOX1 and 18S primer sets were from TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). HMOX1 was normalized to the expression level of 18S for each sample. Relative quantification was acquired by comparative C_T method.

2.8. Polyacrylamide gel electrophoresis and western blotting

Protein was extracted from HUVEC grown on gelatin-coated 12-well multiplates after incubation with CDDO-Im (200 nM), CAPE (20 μM) or 0.1% DMSO for 6 h by addition of 50 μl of lysis buffer (NOVEX, San Diego, CA, USA) containing 10 mM tris (carboxyethyl) phosphine hydrochloride (Sigma, St Louis, MO, USA). Fifteen microliters, containing approximately 5 μg of protein, were run on NuPage 4–12% bis-tris gels (Invitrogen) and then transferred to a nitrocellulose membrane (Invitrogen). After blocking in 0.2% I-Block (Tropix, Bedford, MA, USA), 0.1% Tween-20 (Sigma) and 0.1% thimerosal (Sigma) in PBS, the blots were incubated with a rabbit anti-human HO-1 antibody (Stressgen Biotechnologies Inc., Vancouver, BC, USA; 1:5000) for 2 h. Rabbit ABC alkaline phosphatase reagents (Vector Laboratories, Burlingame, CA, USA) were used to label the bands and the alkaline phosphotase visualization was accomplished with nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl phosphate (Invitrogen) till the bands developed. Quantitative analysis was performed with NIH Images (NIH, USA) on blots scanned into the computer.

2.9. Statistical analysis

Data are presented as the mean plus standard deviation. Differences among groups were analyzed using one-way analysis of variance followed by post hoc tests of Tukey or Games-Howell

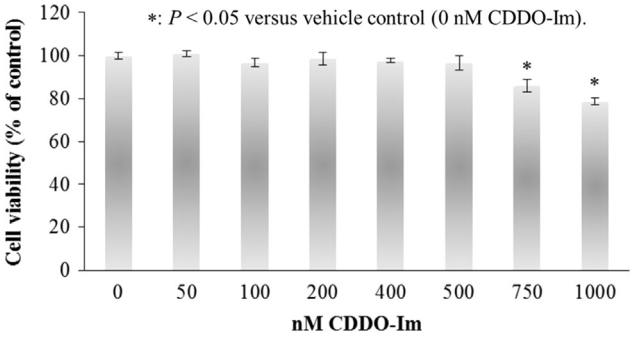

Fig. 1. Cytotoxicity of CDDO-Im to HUVEC. Cell viability shown as percent of vehicle control and values presented as means ± standard deviations ($n=3$). Cell viability less than 90% was considered toxic. CDDO-Im at 750 and 1000 nM were considered toxic.

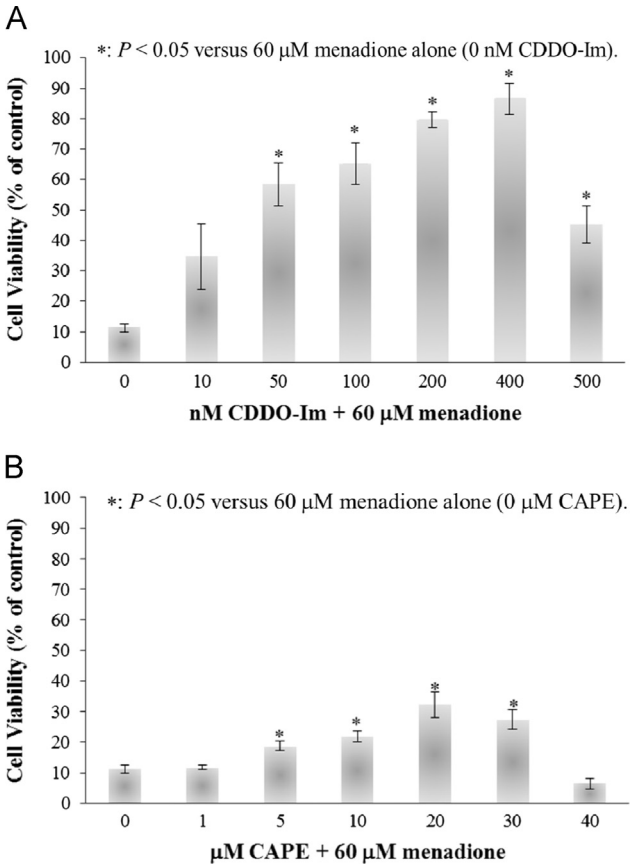

Fig. 2. Cytoprotection of CDDO-Im (A) and CAPE (B) against menadione-induced cytotoxicity in HUVEC. Cell viability is shown as percent of vehicle control and values presented as means ± standard deviations ($n=3$). CDDO-Im and CAPE showed a dose-dependent cytoprotection of HUVEC from 60 μM menadione-induced oxidative stress. CDDO-Im at 200 nM recovered HUVEC significantly to about 80%, whereas CAPE at 20 μM only salvaged around 30% HUVEC compared to control group.

for multiple comparisons through SPSS statistical software. A difference of P value <0.05 was considered significant. All experiments were performed at least 3 times and a representative experiment is presented.

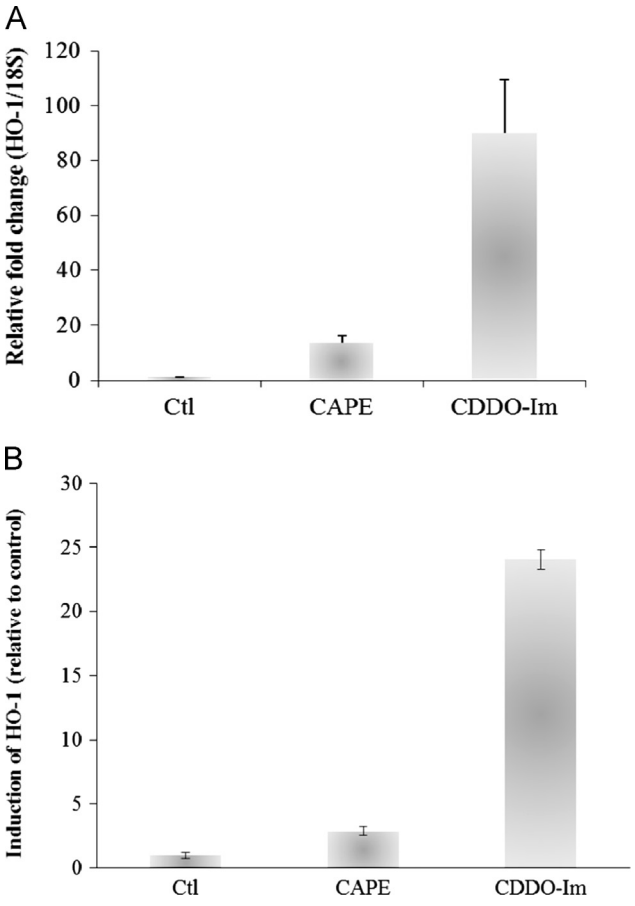

Fig. 3. Comparison of heme oxygenase induction in HUVEC at transcriptional level (A) and translational level (B) by CAPE and CDDO-Im. HMOX1 mRNA was induced up to 90 fold by 200 nM CDDO-Im within 6 h compared to a 13-fold increase following 20 μM CAPE treatment. In addition, HO-1 protein was induced up to 24 fold by 200 nM CDDO-Im within 6 h compared to a 3-fold increase following 20 μM CAPE treatment.

3. Results

3.1. Cytoprotection of CDDO-Im compared to CAPE against oxidative stress in HUVEC

To evaluate the effects of CDDO-Im on cytoprotection against oxidative stress, a model of oxidative stress induced by menadione-generated reactive oxygen species was developed as previously described (Wang et al., 2008). Cytotoxicity of CDDO-Im in HUVEC is shown in Fig. 1. Because 750 nM of CDDO-Im resulted in more than 10% cell death, the maximum dose of CDDO-Im used for cytoprotection testing was 500 nM. It was determined that a dose of 60 μM menadione reduced HUVEC viability to about 10%. CDDO-Im protected HUVEC against menadione-induced oxidant injury in a dose-dependent manner (Fig. 2A). To compare the cytoprotective effect of CAPE to CDDO-Im, HUVEC were treated with various doses of CAPE under the same extent of oxidative insult (60 μM menadione). The results showed that CAPE provided some cytoprotection to HUVEC in a dose-dependent manner (Fig. 2B). CAPE at 20 μM exhibited maximum protection of HUVEC with only about 30% recovery. On the other hand, CDDO-Im at 200 nM protected HUVEC leading to about 80% cell recovery from menadione toxicity. This result indicated that CDDO-Im on a molar basis was about one hundred times more potent than CAPE as a cytoprotectant.

3.2. Identification of potential cytoprotective targets through microarray analysis

To investigate the mechanism of action and identify common molecular targets involved in the cytoprotective effect of CDDO-Im and CAPE in HUVEC, gene expression profiling with microarray analysis was used to monitor alterations in gene expression. For this analysis, quadruplicate HUVEC cultures were treated with CDDO-Im at an optimal cytoprotective dose of 200 nM and CAPE at 20 μM for 6 h, respectively, and compared to vehicle controls. After processing microarray experiments, BRB Array Tool was used to analyze microarray data. After filtering and normalization of gene expression data, class comparison of CDDO-Im or CAPE to the vehicle-treated control group was performed. Upon the activation of 200 nM CDDO-Im, the expression of 2229 genes was found to be significantly regulated ($P<0.001$ and FDR ≤ 0.001) within 6 h. In addition, the expression of 1186 genes was found to be significantly changed ($P<0.001$ and FDR ≤ 0.001) due to the treatment of 20 μM CAPE for 6 h.

To explore common patterns of gene expression altered in HUVEC under the action of CDDO-Im and CAPE, both gene lists were intersected and 339 genes were commonly altered in their

Table 1
Molecular chaperone genes significantly altered (6 h, fold change >2) by CDDO-Im and CAPE treatment in HUVEC.

Symbol	Entrez gene name	CDDO-Im (200 nM)	CAPE (20 μM)
HSPA1A/HSPA1B	heat shock 70 kDa protein 1A	152.85	2.3
HSPA6	heat shock 70 kDa protein 6 (HSP70B')	103.07	
DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4	83.56	
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1	20.3	
HSPH1	heat shock 105 kDa/110 kDa protein 1	12.1	
HSPB8	heat shock 22 kDa protein 8	8.86	
HSP90AA1	heat shock protein 90 kDa alpha (cytosolic), class A member 1	4.74	
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	4.21	
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	4.19	10.46
HSPA8	heat shock 70 kDa protein 8	3.44	
DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	3.27	24.13
HSPD1	heat shock 60 kDa protein 1 (chaperonin)	2.99	
HSP90AB1	heat shock protein 90 kDa alpha (cytosolic), class B member 1	2.28	
DNAJC21	DnaJ (Hsp40) homolog, subfamily C, member 21	2.24	
HSPA9	heat shock 70 kDa protein 9 (mortalin)	2.2	
DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	1.91	4.05
HSP90B1	heat shock protein 90 kDa beta (Grp94), member 1		4.49

expression. Among them, HMOX1, a well-known cytoprotective gene, was induced to about 9 fold by 20 μM CAPE similar to our previous finding (Wang et al., 2008). However, 200 nM CDDO-Im, a dose hundred times lower than CAPE, triggered the induction of HMOX1 up to 43 fold. To validate this particular result from microarray screening, quantitative real-time RT-PCR and western blotting were performed for HO-1 at 6 h. The results confirmed the induction of HMOX1 gene at transcriptional and translational levels (Fig. 3). Interestingly, a number of molecular chaperone genes were highly upregulated by CDDO-Im. These genes were heat shock protein (HSP) and DNAJ related. To take a close look at the transcriptional activation of these chaperone genes by both compounds, we selected and listed all those genes in Table 1. Sixteen chaperone genes were found to be induced by CDDO-Im. Among them, HSP genes HSPA1 (HSPA1A/HSPA1B) and HSPA6 were highly induced up to 150 and 100 fold at 6 h, and DNAJA4 was upregulated to around 80 fold. In comparison, CAPE upregulated 5 chaperone genes including 3 DNAJ genes and 2 HSP genes.

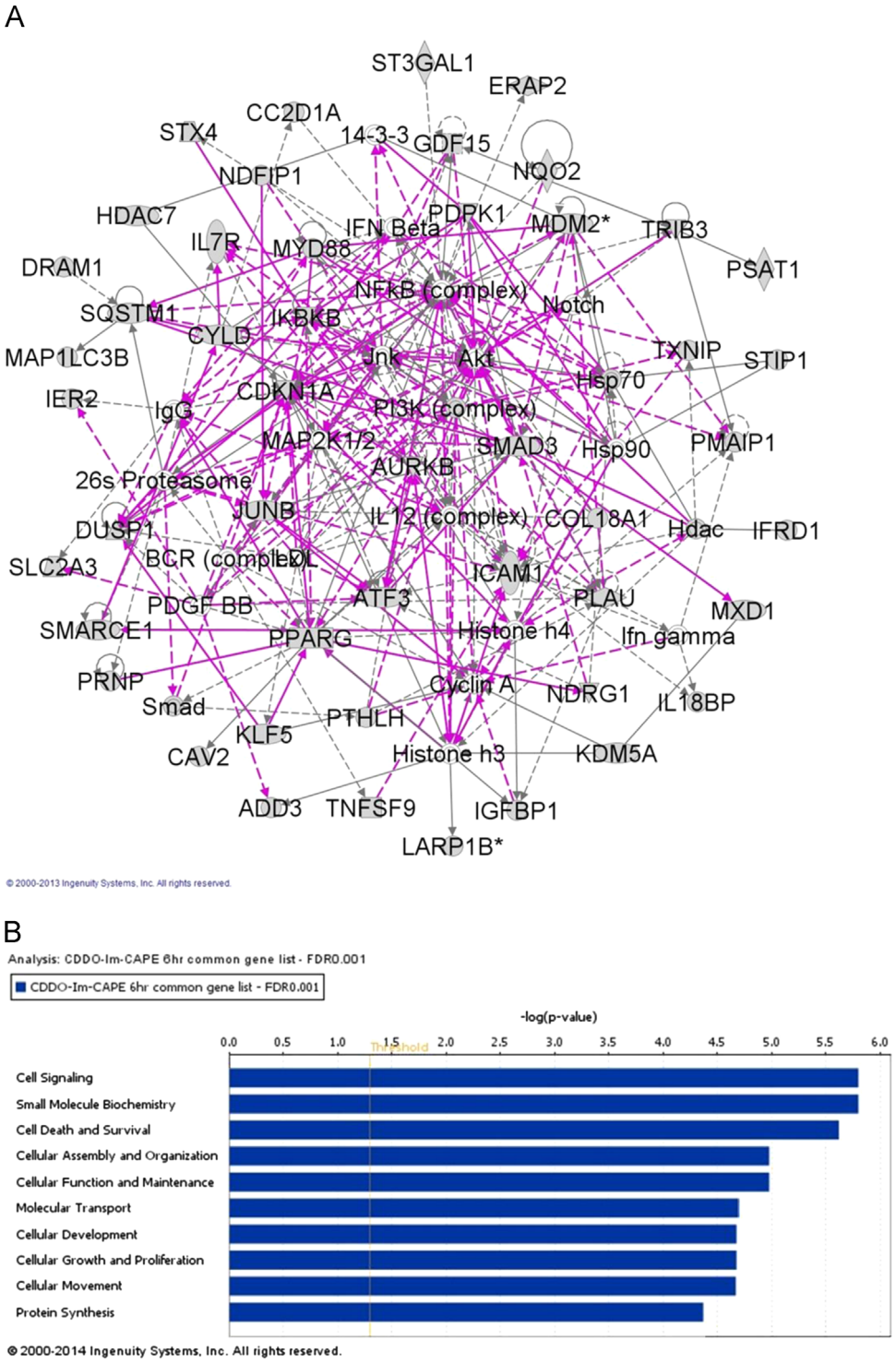

Fig. 4. Top scored network (A) and functions (B) of genes commonly altered by CDDO-Im and CAPE. Network associated functions involve cell death and survival, cellular development, and cellular growth and proliferation, which are among top 10 molecular and cellular functions commonly regulated by both compounds.

3.3. Functional enrichment and pathway analysis by IPA

To better understand the cytoprotective effects of CDDO-Im and CAPE in HUVEC, we used IPA to analyze microarray data sets for enrichment of transcriptional networks, functions, and pathways. The list of 339 common genes was first submitted to IPA to look for common patterns in functions and pathways. Results of network analysis indicate that the most relevant network to our list of genes was enriched in regulatory functions of cell death and survival, cellular development, and cellular growth and proliferation. The corresponding interactions of genes involved in this network were shown in Fig. 4A. In addition, functional analysis found that these functions are among ten molecular and cellular functions (P value ≤ 0.0001) most related to the list of common genes submitted, which was shown in Fig. 4B. Results of pathway analysis identified one canonical pathway of particular interest to us, Nrf2-mediated oxidative stress response, which was highly affected by both compounds (P value = 2.54E-04). Nrf2, a transcription factor, is known to play an important role in cellular defense systems against oxidative stress through activation of an array of antioxidant and

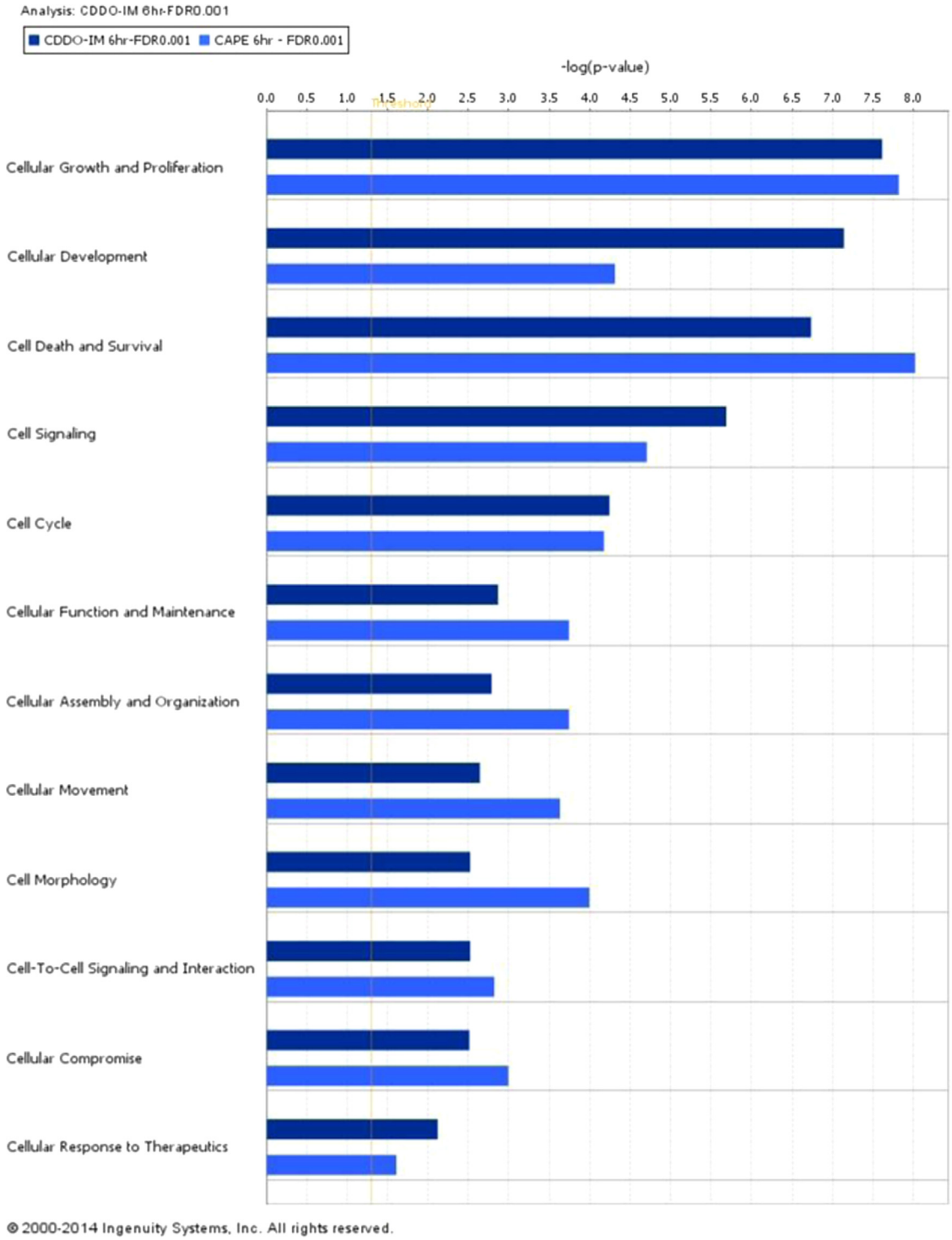

Fig. 5. Comparison of cellular functions most highly enriched in the gene sets altered by CDDO-Im and CAPE.

Table 2
Genes involved in Nrf2-mediated oxidative stress response pathway and modulated by CDDO-Im and CAPE in HUVEC.

Symbol	Entrez gene name	CDDO-Im (200 nM)	CAPE (20 μM)
DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4	83.56	
SCARB1	scavenger receptor class B, member 1	59.59	
HMOX1	heme oxygenase (decycling) 1	42.62	9.38
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1	20.3	
JUNB	jun B proto-oncogene	17.77	3.86
SQSTM1	sequestosome 1	10.3	3.02
HSPB8	heat shock 22 kDa protein 8	8.86	
NQO2	NAD(P)H dehydrogenase, quinone 2	6.89	2.02
FOS	FBJ murine osteosarcoma viral oncogene homolog	6.82	16.34
GCLC	glutamate–cysteine ligase, catalytic subunit	6.78	
VCP	valosin containing protein	6.17	
NQO1	NAD(P)H dehydrogenase, quinone 1	4.48	
MAFG	v-maf musculoaponeurotic fibrosarcoma oncogene homolog G (avian)	4.26	
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	4.21	
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	4.19	10.46
ACTG1	actin, gamma 1	3.34	
DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	3.27	24.13
STIP1	stress-induced-phosphoprotein 1	3.08	4.44
TXNRD1	thioredoxin reductase 1	3.06	
FTL	ferritin, light polypeptide	2.88	2.52
SOD1	superoxide dismutase 1, soluble	2.35	
DNAJC21	DnaJ (Hsp40) homolog, subfamily C, member 21	2.24	
MRAS	muscle RAS oncogene homolog	2.04	
DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	1.91	4.05
PIK3C2B	phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 beta	−4.167	−1.961
ACTA2	actin, alpha 2, smooth muscle, aorta		2.22
BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1		2.44
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3		3.76
RRAS2	related RAS viral (r-ras) oncogene homolog 2		3.1

detoxification enzymes. The activation of this pathway may provide a possible mechanism for the cytoprotective effects of both compounds against oxidant insult in HUVEC as described above.

To explain the difference in cytoprotective effects, we submitted the lists of genes significantly altered by CDDO-Im and CAPE individually and compared their enrichments in cellular function and involvement in canonical pathways. Both compounds affected major cellular functions significantly without notable discrepancies as shown in Fig. 5. When focusing on canonical pathways, in particular Nrf2-mediated oxidative stress response, more related genes and genes with higher up-regulation were found in CDDO-Im than CAPE treatment as shown in Table 2. The canonical pathway representing Nrf2-mediated oxidative stress response from CDDO-Im treatment is shown in Fig. 6.

4. Discussion

Redox homeostasis within cells refers to a well-controlled balance between cellular antioxidant system and reactive oxygen species. Maintenance of this cellular redox homeostasis is important for a number of biological processes since reactive oxygen species at physiological level have been reported to serve as potential signaling molecules mediating cellular proliferation, differentiation, and apoptosis (Zhang et al., 2011). However, when this balance is disturbed as a result of oxidative stress where the overproduced reactive oxygen species are not eliminated by the antioxidant system, the accumulation of excessive reactive oxygen species may result in severe damage to cellular components. Reactive oxygen species can attack cellular components causing oxidative damage to constituents of cell membranes, lipids, proteins, and DNA. Damage to lipids and proteins leads to lipid peroxidation, protein degradation, fragmentation, modification, and inactivation. Although DNA is relatively protected and stable, it can still be attacked by reactive oxygen species resulting in modification of DNA bases, single- and double-strand breaks, and damage to DNA repair systems (Beckman and Ames, 1998; Halliwell and Gutteridge, 2006). Reactive oxygen species generated cellular damage has been implicated in ischemia/reperfusion injury, cardiovascular diseases, neurodegenerative diseases, and cancer (Papaharalambus and Griendling, 2007; Salvemini and Cuzzocrea, 2002; Uttara et al., 2009; Waris and Ahsan, 2006).

A series of synthetic triterpenoid derivatives of oleanolic acid including CDDO and CDDO-Im has been reported to be responsible for promoting cellular control of free radicals through induction of the phase 2 responses (Dinkova-Kostova et al., 2005). In a continuing effort to identify potential agents for ameliorating oxidative stress, we selected CDDO-Im and tested its ability to salvage human endothelial cells from oxidative stress. Our result found that nanomolar concentrations of CDDO-Im were sufficient to keep HUVEC viability close to 80% in an environment of intensive oxidative stress induced by 60 μM menadione. This cytoprotection of CDDO-Im is much more potent than that of CAPE, a natural polyphenolic compound we previously found cytoprotective (Wang et al., 2010, 2008). Under the same extent of oxidative stress, CAPE at micromolar concentrations saved HUVEC up to only 30%. For the next step, we explored the mechanism of cytoprotection by looking for any common cytoprotective factors shared by both compounds and new cytoprotective mediators induced by CDDO-Im, which might provide a possible explanation for the different cytoprotective effects observed.

We investigated the alteration of global gene expression using a microarray approach in order to understand the molecular changes that occur in HUVEC following the treatment of CDDO-Im versus CAPE. We found that 2229 and 1186 genes were statistically significantly altered in expression in HUVEC after 6 h treatment with CDDO-Im and CAPE at optimal cytoprotective doses of 200 nM and 20 μM, respectively. Of these genes, 339 were altered by both compounds, which may suggest a common mechanism of cytoprotection. Out of these 339 genes, HMOX1 was found to be one of them. HMOX1 is a well-known cytoprotective gene, and its protein product HO-1 is now included in the phase 2 enzyme systems. Classical phase 2 enzymes such as glutathione S-transferases and UDP-glucuronosyltransferases are used to conjugate xenobiotics with endogenous ligands like

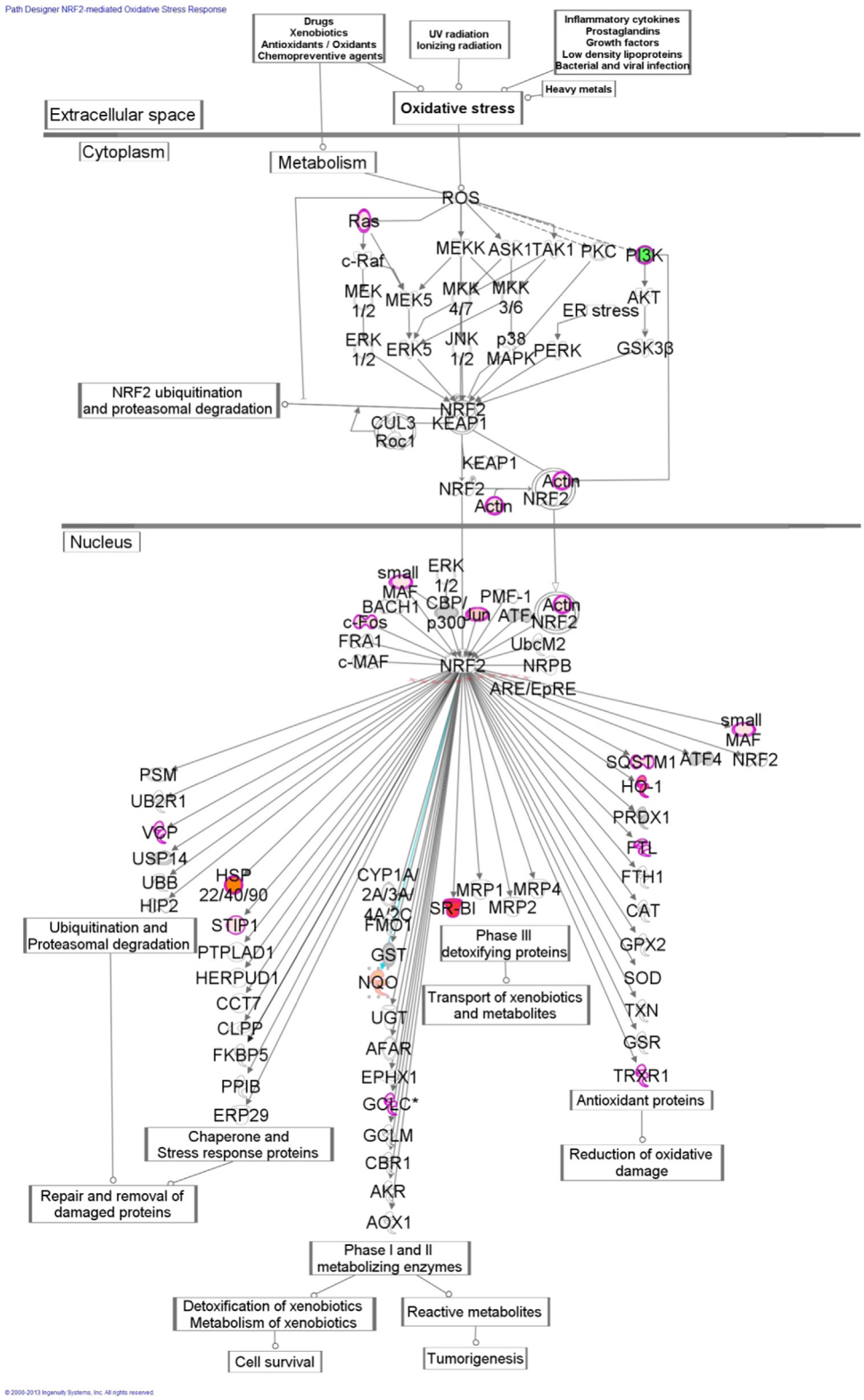

Fig. 6. Nrf2-mediated oxidative stress response pathway from IPA analysis of CDDO-Im gene set. Genes up- and down-regulated are in red and green, respectively.

glutathione and glucuronic acid. This conjugation reaction leads to more easily excretable products. Currently, the list of phase 2 enzymes has been expanded to include enzymes regulated through the antioxidant responsive element and enzymes that lead to cytoprotection against oxidative stress (Talalay, 2000). CDDO-Im was reported to be a strong inducer of HMOX1 in different cell lines at a similar range of nanomolar concentrations (Heiss et al., 2009; Liby et al., 2005; Zhang et al., 2012). Our results confirmed this. It was also shown that HMOX1 was highly up-regulated by CDDO-Im, up to 43 fold in HUVEC, which is much higher than HMOX1 induction by CAPE (9 fold). The results from microarray analysis were confirmed at both transcriptional and translational levels. Much higher levels of expression of HMOX1 mRNA and protein were observed in the CDDO-Im treated group than CAPE treated group. Our previous study showed

that HMOX1 was induced by CAPE, and this likely plays an important role in its cytoprotection of HUVEC. The much higher upregulation of HMOX1 by CDDO-Im may contribute at least in part to its greater cytoprotective effect than CAPE.

In addition to the upregulation of HMOX1, CDDO-Im was found, for the first time to our knowledge, to strongly induce genes of the HSP family, especially HSPA1, HSPA6, and DNAJA4. Heat shock proteins, a highly conserved class of stress response proteins, function as molecular chaperones to prevent protein aggregation, correct protein misfolding, and promote protein refolding. The functions of HSP are crucial to the maintenance of cellular homeostasis by controlling cellular stresses such as endoplasmic reticulum (ER) stress and oxidative stress (Kalmar and Greensmith, 2009; Schroder and Kaufman, 2005). During ER stress, HSP are expressed through activation of pathways called unfolded protein responses (UPR). UPR can also trigger downstream antioxidant responses, which provides a line of defense against oxidative stress. When cells are exposed to oxidative stress, a cascade of events occurs including change in cellular redox balance, oxidation of cell lipid proteins, and protein aggregation and misfolding. As a consequence, inflammation and apoptosis pathways are activated, eventually leading to failure of normal cell function and cell death. However, the induction of HSP can intervene with these events and rescue cells from stress-induced programmed death. For example, heat shock protein Hsp33 is activated due to a change in redox state within bacteria cells (Jakob et al., 1999). Its homologue in eukaryotic cells, Hsp32, was reported to be induced through redox-sensitive transcription factors in response to redox change (Calabrese et al., 2004). Interestingly Hsp32 is also referred to as HO-1. Therefore, HO-1 is considered one of the HSP which enhances the cytoprotective potential of heat shock proteins against oxidative stress. In addition, some HSP such as Hsp70 can bind to apoptosis mediators such as apoptosis protease activating factor-1 and thereby prevent cell death from occuring (Saleh et al., 2000). The anti-apoptotic effect of HSP appears to be independent of their function as molecular chaperones. Upregulation of Hsp70 was also found to reduce inflammatory response possibly through interaction with transcriptional factor, nuclear factor kappa B (Malhotra and Wong, 2002). Anti-inflammatory properties of HSP may contribute to the overall cytoprotective capacity of HSP against oxidative stress. Compared to CDDO-Im, CAPE induced fewer HSP and to a less extent. For example, HSPA1 was upregulated 150-fold upon action of CDDO-Im but only 2-fold under CAPE treatment. The potential cytoprotective role of HSP against oxidative stress could be an additional reason that CDDO-Im, a more potent HSP inducer, protected HUVEC against oxidant insult much better than CAPE.

To identify the cellular phenotypes most significant to the alteration of our gene expression profiles and further understand the corresponding impact of these changes in the content of well-characterized pathways, IPA was used to analyze the microarray data. For the purpose of extracting common patterns between CDDO-Im and CAPE, common genes expressed were analyzed. The results indicate that the most highly-regulated cellular functions include cell signaling, cell death and survival, cellular function and maintenance, cellular development, cellular growth and proliferation, and protein synthesis, which is in agreement with the major functions of the top network generated out of the set of common genes. In addition, canonical pathway analysis pointed out that the Nrf2-mediated oxidative stress response pathway was highly up-regulated. The Nrf2 pathway mediates the expression of an array of genes involved in drug metabolism and transport, antioxidant defense, and oxidant signaling (Ma, 2013). It is considered a useful strategy for detoxification that cells apply in response to challenges from endogenous and exogenous oxidants, electrophiles, and toxicants. Activation of this pathway was reported to facilitate the induction of HMOX1 (Heiss et al., 2009; Liby et al., 2005; Zhang et al., 2012). In a continuing effort to address the differential cytoprotective ability of CDDO-Im and CAPE, a core analysis of individual data set and follow-up comparison were performed in IPA. The significant top cellular functions were found to be similar between groups of CDDO-Im and CAPE. However, Nrf2-mediated oxidative stress response pathway was much more significantly activated in the case of CDDO-Im with more genes involved and higher level of gene upregulation.

In summary, CDDO-Im was identified as a more potent cytoprotectant than CAPE in protecting human endothelial cells from oxidative stress. Both compounds were found to induce HMOX1, affect similar cellular functions, and regulate the Nrf2-mediated oxidative stress response pathway, which may provide a common mechanism behind this cytoprotection. The results suggest that the greater cytoprotection observed by CDDO-Im could possibly be due to (1) stronger induction of HMOX1; (2) more intensive activation of Nrf2-mediated oxidative stress response pathway; and (3) upregulation of the molecular chaperone HSP family. This is the first study to report that CDDO-Im induces HSP to our knowledge, which is useful in further understanding the beneficial effects of this compound. CDDO-Im may serve as an inducer of preconditioning of ER and oxidative stresses to activate unfolded protein responses, which in turn induce the expression of HSP and trigger Nrf2-mediated antioxidant responses in particular the upregulation of HMOX1.

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